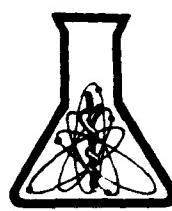


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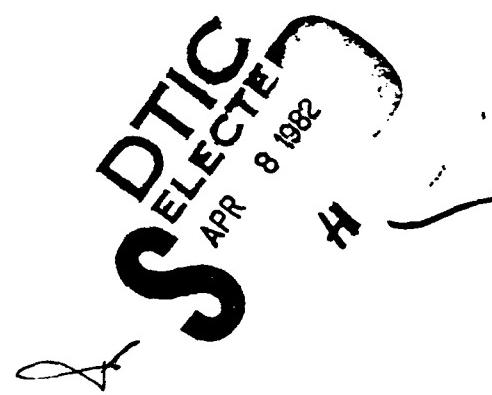
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Structural models of the nicotinic acetylcholine receptor and its toxin-binding sites

H. R. Guy



DEFENSE NUCLEAR AGENCY
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Research was conducted according to the principles enunciated in the "Guide for the Care and Use of Laboratory Animals," prepared by the Institute of Laboratory Animal Resources, National Research Council.

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20. ABSTRACT (continued)

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Structural Models of the Nicotinic Acetylcholine Receptor and Its Toxin-Binding Sites¹

H. Robert Guy^{2,3}

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*Models of the protein structure of agonist-, competitive antagonist-, and snake neurotoxin-binding sites were designed using the sequence of the first 54 residues of the acetylcholine receptor (AChR) α subunit from *Torpedo californica*. These models are based on the premise that the N-terminal portions of the subunits form the outermost extracellular surface of the AChR and that agonists bind to this portion. The models were developed by predicting the secondary structure of the α -subunit N-terminal segment from its sequence, then using these predictions to fold the segment into tertiary structures that should bind snake neurotoxins, agonists, and antagonists. Possible gating mechanisms and quaternary structures are suggested by the proposed tertiary structures of the subunits. Experiments are suggested to test aspects of the models.*

KEY WORDS: acetylcholine receptor; molecular model; protein structure; snake neurotoxin; cholinergic agonists; cholinergic antagonists.

INTRODUCTION

The electrical activity of nerves and muscles is regulated by a variety of voltage- and transmitter-activated proteins that form ion channels through the cellular membranes. A major unresolved problem in neurobiology is the molecular structure of these channels and their associated receptors. It is generally recognized that a precise knowledge of these structures would be invaluable in understanding the functional mechanisms of the nervous system and the ways that various drugs and toxins alter

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these mechanisms. Unfortunately, there are two severe impediments to determining membrane channel structures: (1) most membrane channels are difficult to isolate in sufficient quantities and purity to determine their amino acid sequences, and (2) it is difficult to crystallize membrane proteins in the manner required for X-ray diffraction analysis. Nicotinic acetylcholine receptors and their associated channels (AChR complexes) have been studied extensively. Because of the abundance of AChR complexes in the electric organs of electric rays and eels, considerable progress has been made in isolating them and analyzing their structures. The sequence of the first 54 residues of each of the subunits from *Torpedo californica* AChR complexes has been determined recently (Raftery *et al.*, 1980). Since X-ray data of AChR complex crystal may not be available in the foreseeable future, alternative approaches may have to be used to analyze the data that are available.

The models presented here were developed by using the sequence of the N-terminal portions of the α subunit to design structures that should be energetically stable and that should bind the potent snake neurotoxins and the most potent of the small competitive inhibitors: alloferin. The models are consistent with the available structural data for the AChR complex and suggest mechanisms by which activation occurs. Both models are intended as working hypotheses that can be used to design experiments to eliminate the ambiguities of the model. The models also serve as a method of integrating the various data on the structure and function of the AChR complex into a coherent picture.

EXPERIMENTAL DATA

Structural Data

Before proposing detailed structures for the N-terminal segments, it is necessary to describe the data and a nonspecific model of the entire AChR structure on which

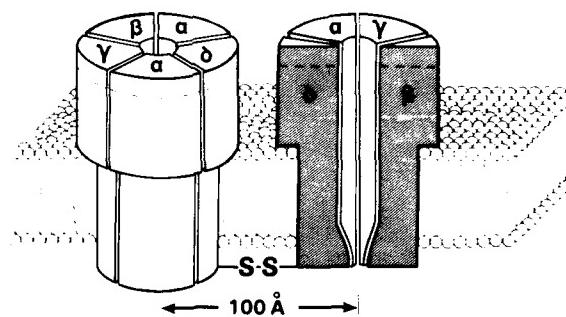


Fig. 1. Nonspecific model of an AChR dimer. A cross section of a monomer is shown on the right. The order of subunits is arbitrary. The models presented in subsequent figures of the N-terminal segments of the subunits represent a top view of the portions of the subunits above the dashed lines. See text for details.

the detailed models are based. A schematicized drawing of the nonspecific model is shown in Fig. 1. Most of the structural data described here were obtained from AChR complexes of the electric organ of *Torpedo californica*. These AChR complexes are comprised of four types of subunits, called α , β , γ , and δ with apparent molecular weights of about 40,000, 50,000, 60,000, and 65,000, respectively (for review, see Heidmann and Changeux, 1978; Raftery *et al.*, 1979). The stoichiometry of the subunits is $\alpha_2\beta\gamma\delta$ (Raftery *et al.*, 1980). These subunits apparently comprise both the agonist receptor and the channel since functional ACh-activated channels have been obtained in preparations in which these were the only subunits present in significant quantities (Gonzalez-Ros *et al.*, 1980; Moore *et al.*, 1979; Nelson *et al.*, 1980; Schindler and Quast, 1980). Studies with cell-free synthesis of AChR subunits suggest that each of the subunits spans the membrane and that the N-terminal ends are on the extracellular side of the membrane (Anderson and Blobel, 1980).

Electron micrographs of membrane fragments that are enriched in AChR complexes indicate the presence of asymmetric "rosette" or "doughnut"-shaped structures that are about 85 Å in diameter and have a center pit or hole of about 20-Å diameter (see Fig. 7) (Ross *et al.*, 1977; Zingsheim *et al.*, 1980). The length of the protein perpendicular to the membrane is about 110 Å, with a 50-Å segment extending from the extracellular membrane surface and a 15-Å segment extending from the intracellular surface (Ross *et al.*, 1977). A calculation of the volume of the rosette structure indicates that its molecular weight is about 255,000 (Klymkowsky and Stroud, 1979). This and the stoichiometries described above indicate that each rosette is comprised of two α , one β , one γ , and one δ subunits. In *Torpedo californica*, the AChR complexes appear to form dimers which are joined by a disulfide bridge between two δ subunits (Raftery *et al.*, 1979). This disulfide bridge appears to be near the C-terminal end of the δ subunits (Oswald *et al.*, 1980) and is thus probably on the inside of the cell.

X-ray diffraction studies of AChR-rich membrane fragments suggest the presence of two large structures: one with a repeat distance of 5.2 Å and a length of 80 Å that probably corresponds to α helices oriented perpendicularly to the membrane, and one with a repeat distance of 6.3 Å and a length of 90 Å that may be due to a β structure (Ross *et al.*, 1977). Circular dichroism and infrared spectroscopy studies of isolated AChR complexes from various preparations suggest that they are comprised of 34% α helices and 29% β sheets (Moore *et al.*, 1974). However, the isolation process may have altered the conformations. Sequences of the N-terminal portions and the total amino acid compositions of all the subunits are very similar (Raftery *et al.*, 1980; Vandlen *et al.*, 1979; Lindstrom *et al.*, 1979). These compositional similarities suggest that the subunits evolved from the same protein and that their overall structures are similar.

The data described in this section support a model in which the AChR complex is comprised of five structurally similar subunits (two α , one β , one γ , and one δ) that stack next to each other so that a channel forms between the subunits when the AChR is in the open conformation. The N-terminal portions for which the sequence is known probably form part of the extracellular domain that binds agonists and competitive inhibitors.

Binding of Agonists and Antagonists

The literature on the binding of various agonists and antagonists is rather confusing and often difficult to evaluate (for review, see Heidmann and Changeux, 1978). In addition to several potential sources of experimental error, the studies are complicated by the fact that the AChRs may have a number of different conformations that bind the drugs with different affinities and possibly by different mechanisms. Affinity-labeling experiments, in which an agonist is covalently bound to a sulfhydryl group on the AChR, suggest that the agonist-binding site is on the α subunit (Karlin *et al.*, 1976). Very potent neurotoxins from the venoms of elapid and hydrophid snakes also bind to the α subunit. Agonists inhibit the binding of the snake neurotoxins. Some studies indicate that one agonist molecule binds for every neurotoxin molecule (Neubig and Cohen, 1979; Weber and Changeux, 1974), whereas other studies indicate only one agonist-binding site for every two neurotoxins (Raftery *et al.*, 1979; Maelicke *et al.*, 1977). The majority of the binding and dose-response data supports a model in which each AChR has two equivalent snake neurotoxin-binding sites and two nonequivalent agonist-binding and competitive antagonist-binding sites.

The binding of snake neurotoxins is inhibited by the competitive inhibitors alloferin, *d*-tubocurarine (*d*-TC), bezoquenonium and hexamethonium. Neubig and Cohen (1979) reported that membrane-bound *Torpedo californica* AChRs bind *d*-TC at two nonequivalent sites and that the binding of one *d*-TC molecule at each site inhibits the binding of one snake neurotoxin molecule. However, Maelicke *et al.* (1977) found that solubilized AChRs from *Electrophorus electricus* have only one curare-type binding site for every two snake neurotoxin-binding sites and that the binding of one curare-type competitive inhibitor inhibits the binding of two snake neurotoxin molecules.

Either the presence of apolar compounds or the disruption of the lipid environment of the AChR appears to alter the conformation of the AChR. Detergents reduce the affinity with which reversible agonists bind (Chang and Bock, 1979). When the AChRs are solubilized or when they are in membranes and apolar compounds such as chloroform and ethanol are present, snake neurotoxins bind to the AChRs with biphasic kinetics in a manner that suggests that the two snake neurotoxin sites interact. The neurotoxins bind to normal membrane-bound AChRs at a slower rate and with simple exponential kinetics that indicate no interaction between the agonist-binding sites (Blanchard *et al.*, 1979; Maelicke *et al.*, 1977).

THEORETICAL MODEL

The evidence just described suggests that the N-terminal portion of the AChR subunits forms part of the extracellular soluble domain that binds agonists, competitive inhibitors, and snake neurotoxins. It is feasible that the N-terminal segments are on the most peripheral and exposed portion of the subunit and that the agonists and antagonists bind to this portion. If this hypothesis is correct, one should be able to use the known sequences to design a structure that will bind the agonists and antagonists and that will be consistent with other experimental data.

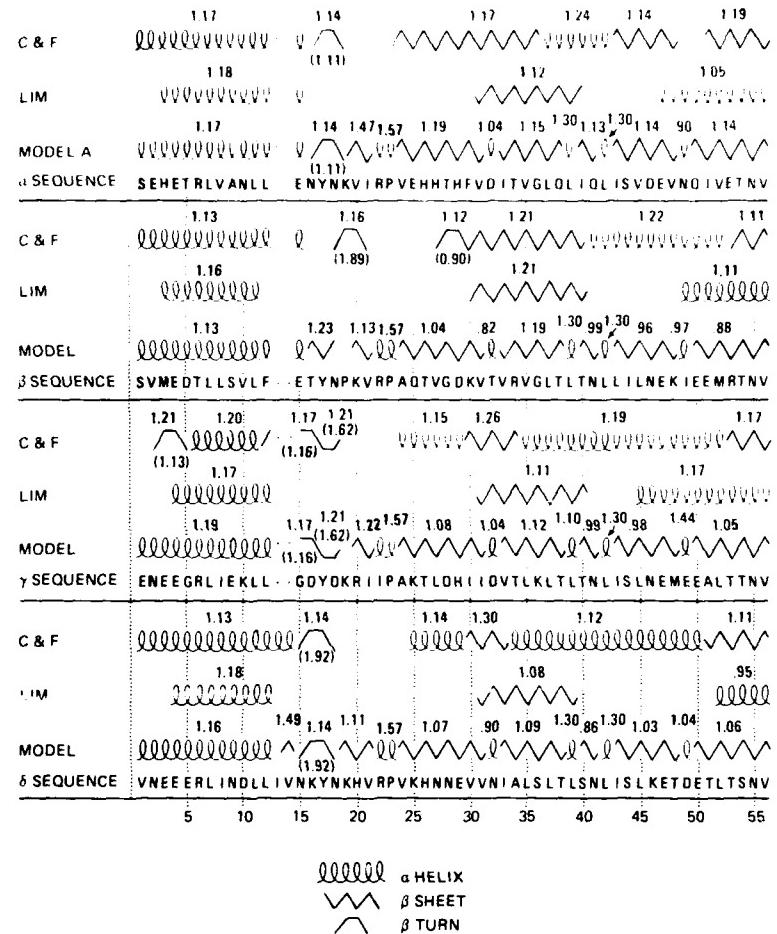


Fig. 2. Comparison of secondary structures predicted by Chou and Fasmen (1978) analysis with Levitt (1979) data base, Lim (1974) analysis, and proposed structure of subunits. Chou and Fasmen structural parameters (P_{α}), (P_{β}), and (P_{γ}) are indicated above appropriate segments. The β -turn parameter $P_t \times 10^4$ is indicated in parentheses. The P_{α} value for Pro-23 was determined from the probability that Pro is one of the first three residues in the N terminal of an α helix (Chou and Fasmen, 1974).

The structures proposed here for the N-terminal segments of the AChR subunits were developed in three stages. First, secondary structures were predicted by using the Chou and Fasmen (1974) and Lim (1974) methods of analysis. Next, tertiary structures for the α subunit were developed by requiring that it bind strongly the crystalline structures of the snake neurotoxins, α cobra toxin and erabutoxin b. Finally, quaternary structures were developed on the basis of interactions among the proposed tertiary structures, the structures of competitive inhibitors that bind to the AChR, and electron microscopy images of AChRs. For the quaternary models, the β ,

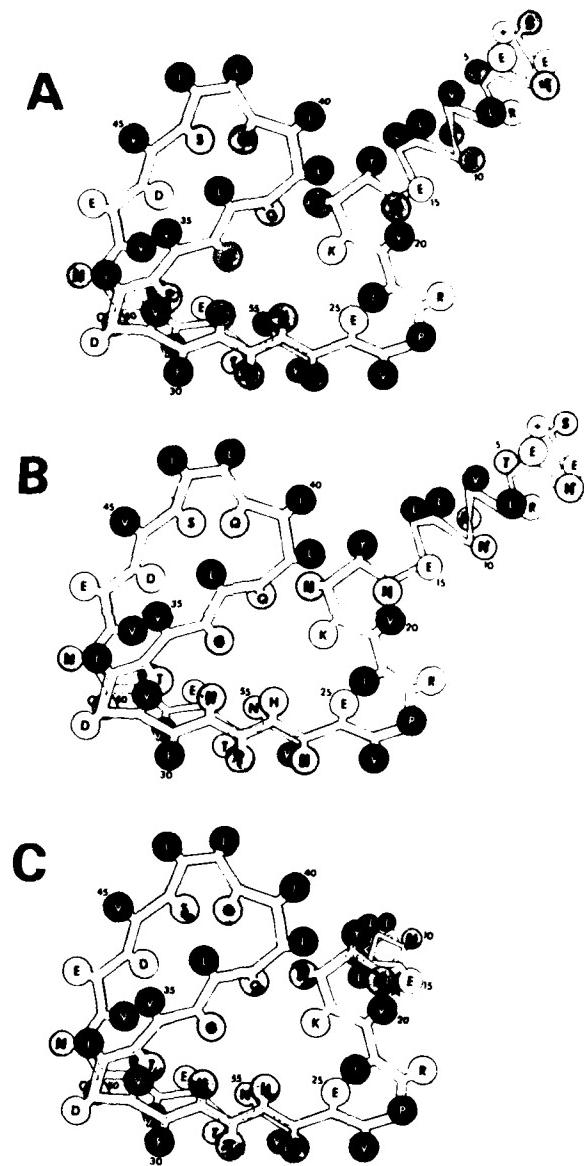


Fig. 3. Three tertiary conformations of the α subunit. Secondary structures of the conformations are the same except for Glu-15 and Asn-16. In A these residues are part of the N-terminal α helix; in B they are second and third residues of a β turn; and in C, Glu-15 is in a random coil, and Asn-16 is the first residue of a β turn. In all figures of protein conformation in this paper, each amino acid side chain is placed in one of three categories according to the free energy change, ΔF , associated with their transfer from a polar to a nonpolar environment (Edelstein *et al.*, 1979). Residues [Cys (C), Pro (P), Met (M), Ile (I), Leu (L), Tyr (Y), Phe (F), Trp (W)] that have a ΔF value greater than 1.0 kcal/mol are classified as apolar and are

γ , and δ subunits were given tertiary backbone structures similar to that of one of the α -subunit models. Different tertiary and quaternary structures were considered in an attempt to account for the conformational changes during activation and desensitization of the AChRs.

Secondary and Tertiary Structures

The most commonly observed structures in proteins are α helices, β sheets, and β turns. Several methods have been developed for using the sequence of a protein to predict which residues will be in each of these type conformations (for review, see Chou and Fasmen, 1978; Sternberg and Thornton, 1978).

In the Chou and Fasmen analysis, each residue has a conformational parameter, P_i , for each of the three secondary structures. A P value greater than 1.0 indicates that the residue occurs in the particular structure more frequently than in a total protein composition. An α helix is predicted for six or more residues when the average α conformational parameter, $(P)_\alpha$, is greater than 1.03, and a β sheet is predicted when $(P)_\beta > 1.05$. A β turn is predicted for a tetrapeptide when its $(P)_\beta < 1.0$ and the factor p_t , based on the product of the frequency of occurrence of each residue in each of the four positions of the β turn, is greater than 0.75×10^{-4} . The predictions of the Chou and Fasmen analysis and the values of the conformational parameter for the four AChR subunits are shown in Fig. 2. The data base on which the method depends has been enlarged recently (Levitt, 1979). The enlarged data base is used here.

An alternative approach of predicting α and β segments is to analyze the distribution of polar and apolar residues within the sequence. Soluble proteins tend to have polar groups on the exterior of each domain and apolar groups buried in the hydrophobic core. Using this principle, Lim (1974) developed a method of predicting the α -helix and β -sheet segments. The predictions of this method are shown in Fig. 2. The α helices and β sheets of the AChR subunits predicted by this analysis should be on the surface of the subunit. The α helices predicted by the Lim analysis are amphipathic; i.e., the hydrophobic side chains are clustered on one side of the helices. These hydrophobic residues should be in contact with the hydrophobic core of the subunit. The predicted β sheet formed by residues 31 to 39 in each subunit has a hydrophobic residue in every other position, so that one side of the β sheet has all hydrophobic residues. These residues should face the hydrophobic core of the protein.

α Subunit. Three possible tertiary structures for the α subunit are shown in Fig. 3. The differences among the secondary structures of the three conformations involve only residue Glu-15 and Asn-16. In conformation A these residues are the last two residues of the N-terminal α helix, whereas in conformation B they are in the second and third positions of a β turn. The transition of these residues from the β turn to the α helix moves the α helix about 2.2 Å closer to the rest of the subunit, lowers the α helix slightly, and changes its orientation slightly. In conformation C the N-terminal α helix has hinged about the Glu-15 and Asn-16 residues so that the α helix is oriented

represented by filled circles. Residues [Ser (S), Asn (N), Gln (Q), Gly (G), Thr (T), His (H), Ala (A)] that have a ΔF value between -0.3 and 0.5 kcal/mol are classified as indifferent and represented by shaded circles. Residues [Asp (D), Glu (E), Lys (K), Arg (R)] that have ΔF values more negative than -2.5 kcal/mol are charged and represented by open circles. The N-terminal amine groups are indicated by a '+' symbol.

perpendicular to the plan of the membrane. Possible implications of these different conformations on gating mechanisms are discussed later.

The secondary structure of conformation A is compared in Fig. 2 to the structures predicted by the Chou and Fasmen and Lim analyses. The agreement with the Chou and Fasmen analysis is very good. The only residues that are in conformations not predicted by the Chou and Fasmen analysis are Asp-31, the six residues from Leu-37 to Leu-42, and Gln-50. Most of the residues that are in conformations not predicted by the Chou and Fasmen analysis are in conformations consistent with the Chou and Fasmen parameters. In the model, all of the residues from Val-24 to Val-56 are in a β -sheet conformation except for Asp-31, Leu-39, Leu-42, and Asn-49. The ϕ , ψ angles about the α carbons of these residues are similar to those of α -helix residues. All of these residues have a P_{α} value greater than their P_{β} value. Val-20 and Ile-30 and Ile-40 have a β -sheet conformation consistent with their high P_{β} values; however, the β sheets containing these residues are too short to be predicted by the analysis. The Pro-23 residue is in a conformation similar to one of the initial residues of the N terminal of an α helix. Pro is one of the most commonly found residue in the first three positions of α helices.

The B conformation of the α subunit has a β turn from residues 12 to residue 17. [Using the Raftery *et al.* (1980) numbering, there are no residues in the 13th and 14th positions for the α , β , and γ subunits.] Its $\langle P_{\beta} \rangle$ value of 0.98 is almost high enough to predict the turn; but, the presence of Leu-12 in the first position of the turn makes it too low since P_{α} of Leu is much higher than P_{β} . However, in the model presented here the Leu-12 residue is the last residue of the α helix as well as the first of the β turn. Thus, the β turn of the B conformation is reasonable. The Chou and Fasmen analysis of the γ subunit predicts a β turn in this position.

The main basis for selecting the tertiary structure of the α -subunit residues 15 to 49 is the formation of a binding site for the snake neurotoxins. Venoms from the elapid and hydrophid snakes contain neurotoxins that bind with a high affinity to nicotinic ACh receptor. These neurotoxins have been classified into two categories: the short-chain neurotoxins that have 60 to 62 residues and the long-chain neurotoxins that have 67 to 70 residues. The sequences of over 50 of these neurotoxins have been determined (Karlsson, 1979). Certain positions within these sequences are always occupied by one or a few similar residues. These invariant residues have been classified into two categories: the structurally invariant residues that are important in determining the overall structure of the toxin protein, and the functionally invariant residues that are important for the binding of the neurotoxin to the AChR (Low, 1979).

The crystalline structures of the short-chain neurotoxin, erabutoxin b (Low *et al.*, 1976; Kimball *et al.*, 1979; Tsernoglou and Petsko, 1976), and the long-chain neurotoxin, α cobra toxin (Walkinshaw *et al.*, 1979) have been determined. Nicholson molecular models of the crystalline structure of erabutoxin b and α cobra toxin were constructed. Next, a model of the N-terminal portion of the α subunit was constructed. The Chou and Fasmen and Lim analyses were used as general guidelines for the secondary structure of the α subunit. Attempts were then made to find a conformation of the α subunit that will bind the snake neurotoxins with a high affinity. Proposed binding structures of erabutoxin b and α cobra toxin and the way they bind to the model of the AChR are shown in Fig. 4.

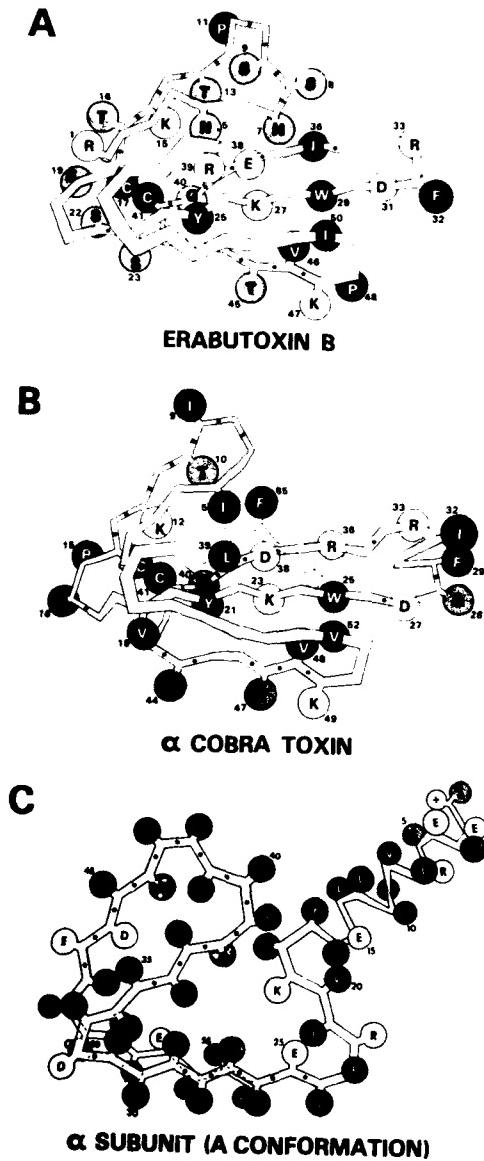


Fig. 4. (A, B) proposed binding structures of erabutoxin b and α cobra toxin. Only those side chains that can interact with the proposed AChR structure are shown. The α cobra toxin structure is the same as the crystal structure (Walkinshaw *et al.*, 1980). The erabutoxin b structure is like the crystal structure (Low *et al.*, 1976; Tsernoglou and Petsko, 1976) except for residues 44 to 48. (C) A conformation of the α subunit. Asterisks indicate backbone polar groups that form H bonds when toxins bind to AChR. Table I indicates which groups of toxins and the α subunit interact.

The proposed α -subunit structures should bind the neurotoxins very strongly. Interactions between the neurotoxins and the α subunit are indicated in Table 1. Erabutoxin b forms 6 salt bridges, 29 hydrogen bonds, and 9 hydrophobic bonds, and α cobra toxin forms 5 salt bridges, 26 hydrogen bonds, and 11 hydrophobic bonds with the α subunit. The structure of α cobra toxin is identical to the crystalline structure; however, the structure of residues 44 to 48 of erabutoxin b has been changed slightly. This change is conservative since it involves only a few residues on the periphery of the toxin and the change converts the erabutoxin b structure to one more like the crystalline α cobra toxin structure. An important feature of the proposed binding

Table 1. Bonds Between Snake Neurotoxins and Model I of the α Subunit^a

	α Cobra toxin	α Subunit	Erabutoxin b	α Subunit
Salt bridges			R-1 K-15 K-27 D-31 R-33 E-15 E-38	E-47 & D-32 D-46 E-25 R-22 R-33 E-15 K-19
Hydrogen bonds			N-5 bb 7-11 H-7 S-8 S-9 bb 12-17 T-13 T-16 S-19 S-22 bb 22-23 S-23 Y-25 N-16 E-38 bb 37-41 bb 39-35 bb 27-24 H-26	Q-41 bb 39-43 N-16 Y-17 Q-41 bb 43-48 Q-41 & S-44 E-47 bb 32-33 or D-32 bb 31-32 bb 30-31 bb 29-30 H-27 & H-29 N-16 Q-38 bb 39-35 bb 28-24 T-45 H-26
Hydrophobic interactions	I-5 I-9 C-14 P-15 V-19 W-25 L-31 L-39 C-41 V-48 I-52 F-65	L-37 I-43 V-35 I-33 V-31 I-21 or V-20 L-11 L-37 V-31 I-21 I-21 I-40	P-11 C-17 I-36 W-29 I-36 C-41 V-46 P-48 I-50	I-43 V-31 V-20 & L-39 V-31 I-21 P-23 I-21

^a Shown in Fig. 4. The bb notation indicates sections of polypeptide backbone that form hydrogen bonds. Other notations same as in Fig. 4. Types of bonds are divided into three categories: salt bridges, hydrogen bonds, and hydrophobic interactions.

scheme is the hydrogen bonds formed between the backbones of the neurotoxins and AChR. Most of these bonds involve the formation of extended β sheets. The postulate that the backbone polar groups of the β sheets of the neurotoxins bind to backbone groups on β segments of the α subunit is very helpful in designing the AChR model since the positions of the backbone groups are much more constrained than those of the side chains.

β , γ , and δ Subunits. For simplicity and because of the homology of the sequences of the four subunits, the β -, γ -, and δ -subunit tertiary structures were made as similar to that of the α subunit as was feasible. The structures of the subunits shown in Fig. 5 are not as consistent with the Chou and Fasmen analysis as is the proposed α -subunit structure. These structures are important in analyzing the quaternary structure of the AChR complex. In that respect, only those regions that interact with adjacent subunits are important for the analysis. These regions are residues 6 to 17 and 39 to 50 for the γ and δ subunits and residues 6 to 22 and 39 to 42 for the β subunit. The other regions could have a conformation more consistent with the Chou and Fasmen and Lim analyses. Those portions that have apolar side chains exposed to the aqueous phase and buried charged groups (e.g., residues 50 and 51 of the β subunit) in the final quaternary model are particularly suspect.

The γ -subunit backbone structure is identical to that of the α -subunit B conformation. The B conformation was selected because the first β turn (residues 12-17) is predicted by the Chou and Fasmen analysis. The δ -subunit conformation differs only in the region surrounding residues Ile-13 and Val-14, which are absent in the other subunits. The β turn of residues 15 to 18 is predicted. The conformation of the β subunit is perhaps the most speculative since its sequence differs most from that of the other subunits. The orientation of its N-terminal α helix downward and conformations of residues 15 to 19 was selected primarily on the basis of the quaternary structure presented in the next section.

Quaternary Structures

The quaternary structures proposed here were designed to satisfy four criteria: (1) the interactions between adjacent subunits must be energetically favorable, (2) the overall structure should account for the binding of agonists and antagonists, (3) there should be at least two conformations to account for activation of the AChR complex, and (4) at least one of the conformations should be consistent with electron microscopy images of membrane-bound AChR complexes.

Interactions Among α , γ , and δ Subunits. The proposed α , γ , and δ tertiary structures have a relatively high number of exposed apolar side chains. These apolar residues would make the structure unstable unless they are buried either by an additional segment of the same subunit or between adjacent subunits. Figure 6 shows how the B-conformation α subunit can form a dimeric complex with the γ subunit in a way that buries many of these apolar residues. Similar complexes between an α and a δ subunit, two α subunits, and a γ and a δ subunit would accomplish the same thing. Most of the apolar residues remain buried if the subunits are placed in the A or C conformations of Fig. 3.

The two subunits that have the most similar sequences are the γ and δ subunits. If the γ subunit forms a dimeric complex with one α subunit, it is thus reasonable to

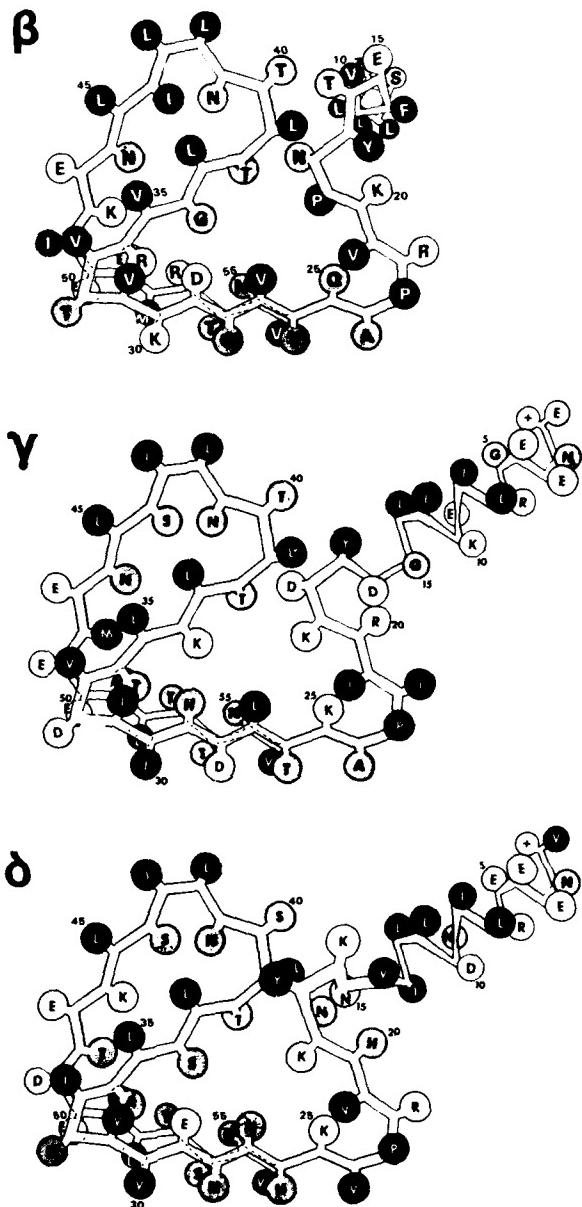


Fig. 5. Postulated tertiary structures of β , γ , and δ subunits. The backbone structure of γ is identical to that of the B conformation of the α subunit. The backbone conformations of residues 20–56 of the β and δ subunits are identical to that postulated for the α subunit. Residues 1–6 of the β subunit are not shown.

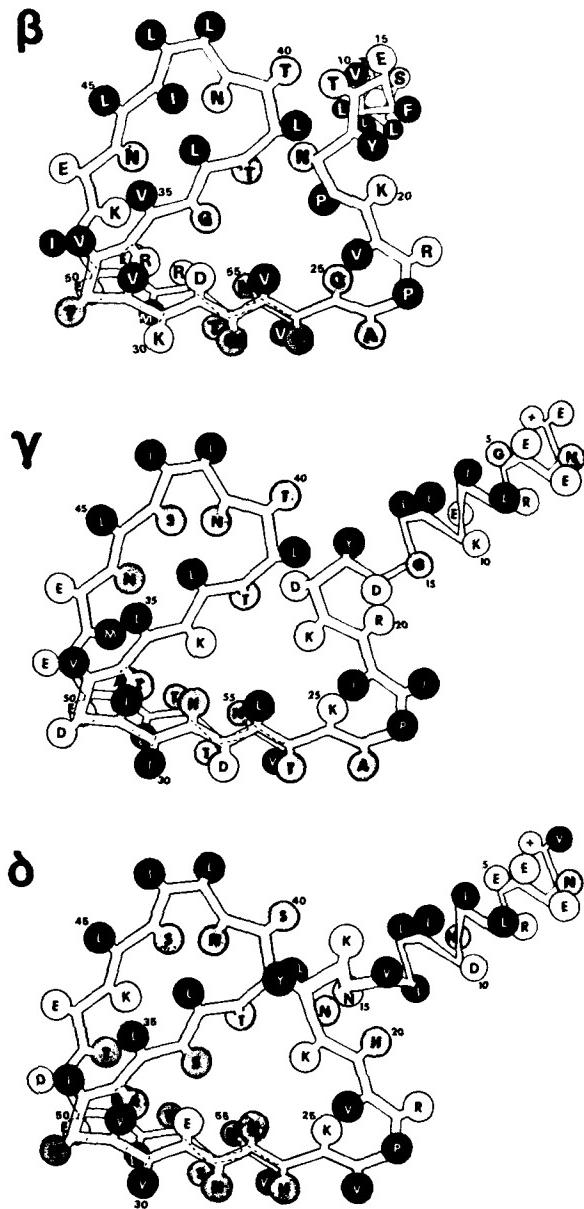


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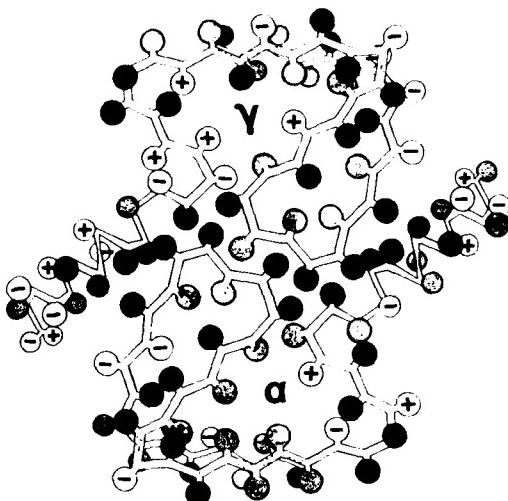


Fig. 6. A dimeric complex of α and γ subunits. Backbones of the subunits are the same. Subunits are positioned so that there is twofold symmetry for backbones. Note that most of the residues at the interfaces between subunits are apolar.

assume that the δ subunit forms a similar dimeric complex with the other α subunit. If this hypothesis is correct, one should be able to position the $\alpha-\gamma$ complex, $\alpha-\delta$ complex, and β subunit next to each other in a way that will account for the binding of agonists and antagonists, the opening and closing of the channel, and the electron microscopy images of membrane-bound AChRs.

Figure 7A shows an electron microscopy image of a membrane-bound AChR from *Torpedo marmorata*. The image has three lightly stained regions that surround a densely stained area. The AChR complex is clearly assymetrical, with the appearance of an opening to one side. Kistler and Stroud (1980) have obtained similarly shaped images from *Torpedo californica*; however, the three lightly stained areas are not as apparent in their images.

The electron micrograph image can be closely mimicked by assuming that one of the lightly stained areas corresponds to the β subunit, that the other two areas correspond to $\alpha-\gamma$ and $\alpha-\delta$ complexes, and that the densely stained area is the entrance to the channel. The complex shown in Fig. 7B is a hypothetical open conformation in which both α subunits have the C conformation of Fig. 3. The N-terminal α helix of the β subunit separates the $\alpha-\gamma$ and $\alpha-\delta$ complexes on one side. This positioning explains why the N-terminal α helix of the β subunit has more apolar side chains than those of the other subunits. There is a gap between the complexes on the side opposite the β subunit. The dimensions of the gap are based on the closed conformation in Fig. 8. The gap could easily be smaller for the open conformation. This gap accounts for the apparent opening on one side of the image of the AChR. The binding of the β subunit to the γ subunit was favored over binding to the δ subunit

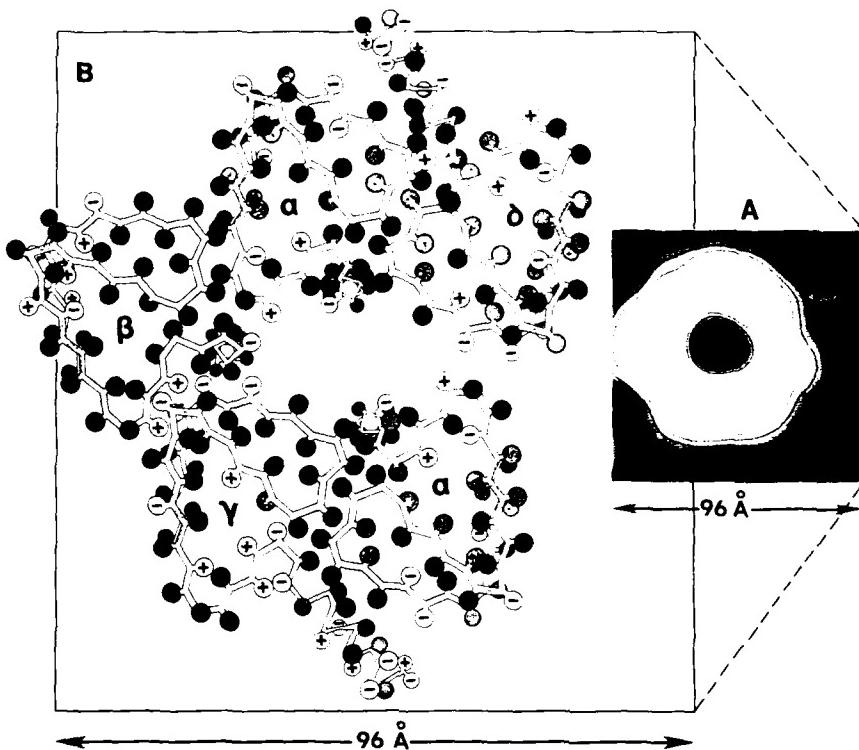


Fig. 7. Comparison of postulated AChR open conformation to electron microscopy image of negatively stained membrane-bound AChRs (reproduced with the permission of Zingsheim *et al.*, 1980). Two of the least stained regions are postulated to correspond to α - γ and α - δ complexes, and the third region to the β subunit. The channel is formed between subunits.

because the δ subunits of two AChR monomers bind together to form the AChR dimer. The presence of a β subunit bound to the δ subunit could interfere with this process.

The closed conformation in Fig. 8 is identical to that of the open conformation except that the α subunit of the α - δ complex has the A conformation in Fig. 3 and the α subunit of the α - γ complex has the B conformation. A difference in the conformation of the two α subunits is required by the asymmetric positioning of the α - γ and α - δ complexes that is suggested by the AChR image. In this model the α helices of the α subunits are held in place by a series of salt bridges between side chains of the two α subunits and the hydrophobic interactions between the α and the γ or δ subunits.

In addition to the asymmetric structures in Figs. 7 and 8, one can easily envision structures in which the two α subunits have twofold symmetry, i.e., they have the same conformations, and one is rotated 180° to the other. Three such structures are shown in Fig. 9. This figure illustrates that activation and desensitization could be due to tertiary and/or quaternary conformational changes. The structure in Fig. 9A is

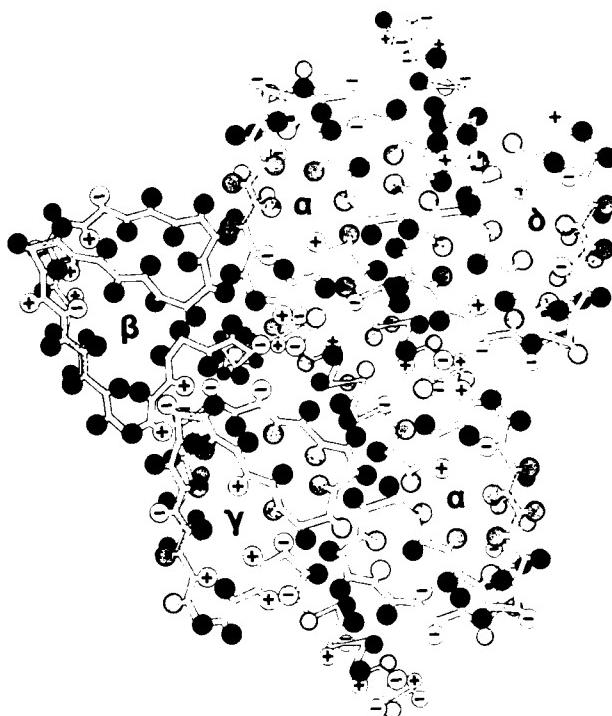


Fig. 8. Postulated closed conformation of AChR. Conformation is the same as the open conformation in Fig. 7 except for the first 14 residues of α subunits.

similar to that in Fig. 8 except that both α subunits are in the A conformation and the relative positions of the subunits have shifted slightly. A tertiary conformation change of the α subunits to the C conformation leads to the open conformation in Fig. 9B. The tertiary structure of this conformation is identical to the structure in Fig. 7B. However, the positions of the subunits are shifted; i.e., the quaternary structure is different. A different type of quaternary change, involving a shift of the α - γ complex, leads to the closed structure in Fig. 9C. It is possible that desensitization is due to this type of conformational change.

It is obvious from the five conformations just described that, with five subunits, one can postulate many different tertiary and quaternary conformational changes that could cause activation and desensitization. Additional data are needed to decide which conformations are most likely. The structures in Figs. 7B and 8 are more consistent with the image of the AChR in Fig. 7A and with reports that the two agonist-binding and antagonist-binding sites differ. However, the structures in Figs. 9A and B are supported by the structures of the snake neurotoxins and the potent competitive inhibitor alloferin (also called alcuronium).

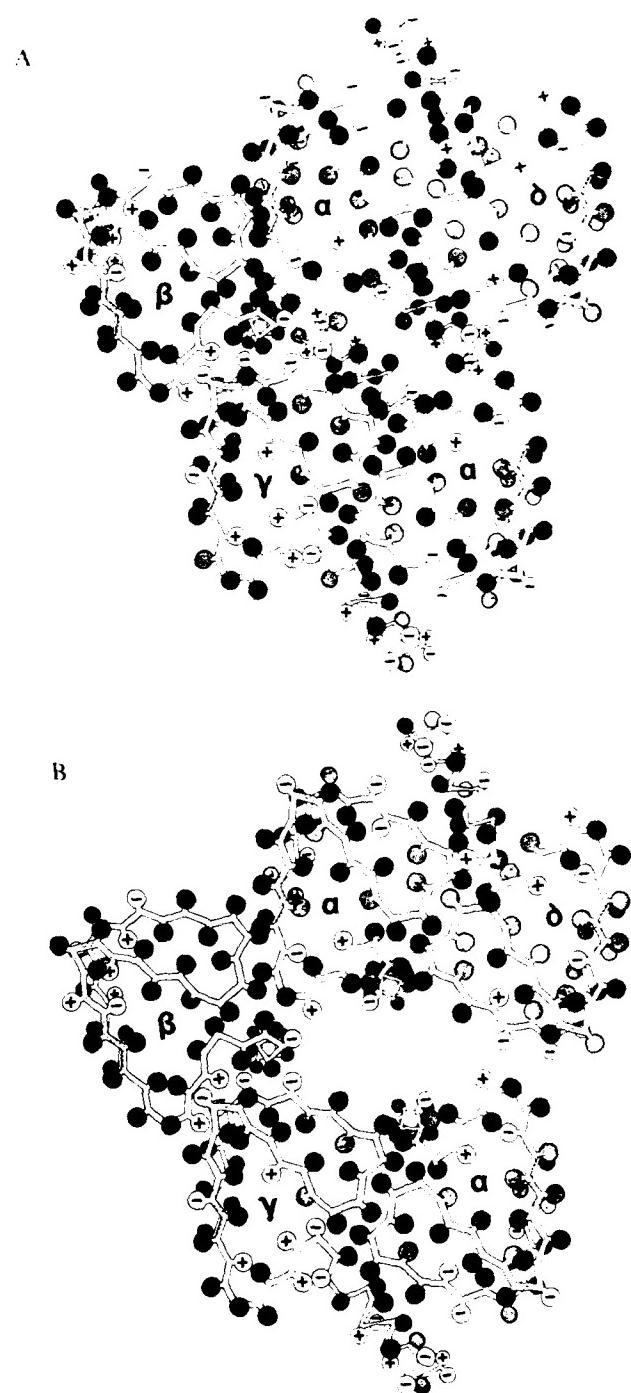


Fig. 9. Conformations in which α subunits have identical conformations and are rotated 180° with respect to each other.

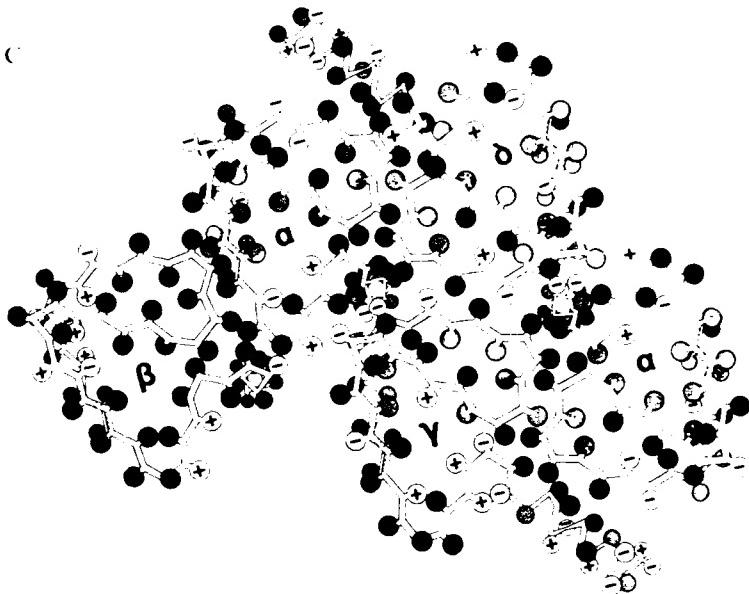


Fig. 9. Continued.

When an α cobra toxin binds to each of the α subunits of the structure in Fig. 9A, the "tail regions" (residues 28–33) of the two toxin molecules meet in a manner that closely mimics the structure of alloferin (Fig. 10). In addition to the interactions with the α subunit described earlier, several side chains of α cobra toxin and erabutoxin b can bind to side chains of the α helix of the adjacent α subunit. Alloferin should bind with a high affinity since each of its positively charged moieties can bind to an α -subunit Glu-15 carboxyl group, its hydroxyl group can bind to the Asn-10 and Asn-16 amide groups, and each aromatic ring can fit between the Leu-7 and the Asn-10 side chains and next to the Ala-9 side chain. The neurotoxins tail segments bind in a similar manner.

The symmetrical closed structure in Fig. 11 should also bind alloferin and a single snake neurotoxin molecule with a high affinity. In addition to the interactions with the α subunits, the apolar Phe-32 side chains of erabutoxin b and Phe-29 side chain of α cobra toxin can fit between the Ile-43 and the Leu-45 side chains of the γ or δ subunit and the positively charged erabutoxin b Lys-47 and α cobra toxin Lys-49 side chains can form salt bridges with the Asp or Glu-49 and Asp or Glu-31 side chains of the γ or δ subunits. Each of the positively charged moieties of alloferin can bind to the Glu-15 carboxyl group, its hydroxyl groups can bind to the amide groups of Asn-16, and its apolar moieties fit next to the apolar Ile-43 and Leu-45 side chains of the γ and δ subunits and the Ala-11, Val-8, and Val-20 side chains of the α subunit. Because of steric hindrance between the tail regions of the neurotoxins, only one neurotoxin molecule can bind at one time. The predicted stoichiometries of this model appear consistent with binding studies to isolated AChRs from *Electrophorus*.

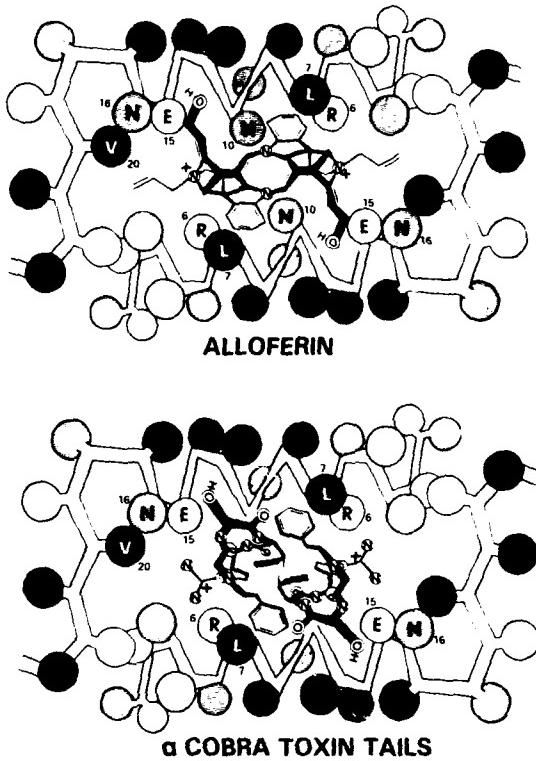


Fig. 10 A comparison of binding of (A) alloferin and (B) two α cobra toxin "tails" (residues 27–33) to the N-terminal α helices of α subunits in the Fig. 9A conformation. Note the similarities in structure of alloferin and cobra toxin tails.

electricus (Maelicke *et al.*, 1977) but are not consistent with studies of membrane-bound AChRs from *Torpedo californica* (Neubig and Cohen, 1979). The biphasic kinetics of neurotoxin binding to isolated AChR (Maelicke *et al.*, 1977) or to membrane-bound AChRs when ethanol and chloroform are present (Blanchard *et al.*, 1979) are also consistent with steric hindrance in the binding of the two neurotoxin molecules. Binding kinetics to normal AChRs indicate no interaction (Blanchard *et al.*, 1979). It is possible that the isolation procedure or the addition of chloroform and ethanol alters the AChR to the conformation in Fig. 11.

There are a number of reasons to suspect that the positively charged moieties of agonists and antagonists bind to the Glu-15 carboxyl group of the α subunit: (1) Tsernoglou *et al.* (1978) and Kimball *et al.* (1979) have suggested that the Arg-33 and Asp-31 side chains of erabutoxin b mimic the structure of ACh and that the guanidium group of the Arg side chain binds to the negatively charged group of the agonist-binding site. In the model presented here, the Arg-33 group binds to Glu-15 of

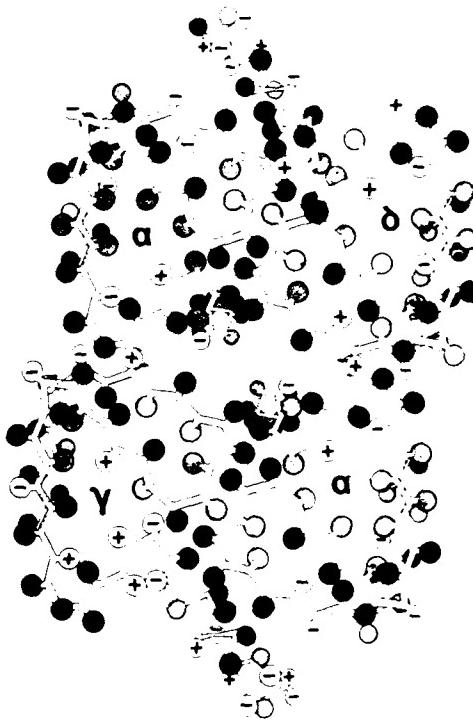


Fig. 11. Conformation that can bind only one snake neurotoxin molecule. The β subunit is not shown and cannot bind in the manner of conformations shown in Figs. 7-10.

the α subunit. Glu-15 is also the residue to which the positively charged moieties of alloferin are postulated to bind. (2) Glu-15 is one of the two residues for which the secondary conformation changes in the proposed mechanism of channel activation. This conformational change could most easily be induced by the binding of the agonist to Glu-15 and Asn-16. (3) Smythies (1980) proposed a model in which the agonist and antagonist sites are located between two α helices formed by the first 21 residues of the α subunits. His model is based on a stereochemical analysis of the binding of agonists and antagonists to the proposed structure. The first 12 residues of the Smythies model is virtually identical to the "closed" conformation proposed here; however, the rest of the structure is entirely different. Because of the similarities of the models, some of the stereochemical arguments of the Smythies model are applicable to the model presented here. One of the agonist-binding sites on the Smythies model involves Glu-15.

If the proposed model is correct, agonist and antagonist should be able bind to the AChR in a manner consistent with experimental findings. Beers and Reich (1970) analyzed the conformation of several nicotinic agonists and antagonists and proposed that the bindings of these agents is due to a coulombic interaction involving the

positively charged alkylammonium moiety and a hydrogen bond to an acceptor group on the agents that is formed 5.9 Å from the center of the charge. All of the agonists (ACh, nicotine, and cytisine) and antagonists (trimetaphan, β -erythrodine, and strychnine) considered by Beers and Reich can bind to the Glu-15 carboxyl group of the postulated closed and open conformations in a manner that allows their acceptor group to form a hydrogen bond with the guanidium group of Arg-6 or Arg-22 or with the amide group of Asn-10 or Asn-16. Using these groups, one can conceive of several ways the agonists and antagonist could bind. For the closed conformation in Fig. 8, one of the α subunits is in the A conformation and the other is in the B conformation. The positively charged moiety of ACh can bind to the Glu-15 carboxy group of A conformation site so that the oxygens of the ACh ester linkage form hydrogen bonds with the Asn-16 and Asn-10 amide groups and the end methyl group sets next to the Leu-11 side chains or it can bind so that the oxygens bind to the Arg-6 or Arg-22 guanidium groups and to the Asn-16 amide group and the end methyl group fits next to the Val-20 side chain. ACh could bind to the same two sites of the B conformation, but the binding would be different. The simultaneous binding of the two ACh oxygens to the Asn-10 and Asn-16 amide groups is not as favorable since the amide groups are farther apart. The binding of the oxygens to the Asn-16 and Arg-22 side chains may be more favorable. In all of the conformations there will be some coulombic interaction between the positively charged moiety of ACh and the Glu-2 carboxyl group.

For an agonist to activate the AChR, it must bind with a higher affinity to the open than to the closed conformation. ACh can bind to the α -subunit C conformation of the open AChR by binding to the same groups, with the exception of Arg-6, as it binds in the closed conformation. The two oxygens can bind simultaneously to the Asn-10 and Asn-16 amide groups or to the Arg-22 and Asn-16 groups. The spacing between the groups and thus the binding would be different. Perhaps more important is the removal of the Arg-6 side chain from the vicinity of the cation binding site. In the open conformation the ACh binding sites have a net negative charge due to Glu-15, Arg-22 of the α subunit, Glu-47 of the γ or δ subunit, and, for one of the sites, Glu-15 of the β subunit. The closed conformation has a neutral charge if one considers only the Glu-15, Arg-22, Glu-2, and Arg-6 side chains. When His-2 is charged, the site is more positive than negative.

Divalent agonists and antagonists also can bind in a number of ways. A possible mechanism by which alloferin binds to the Glu-15 side chains of the two α subunits has already been described. This mechanism is not appropriate for the binding of *d*-tubocurarine (*d*-Tc) to membrane-bound *Torpedo californica* AChRs since it binds to two nonequivalent sites and the binding to one site inhibits the binding of only one snake neurotoxin molecule (Neubig and Cohen, 1979). More likely mechanisms for *d*-Tc binding are to the closed conformations Glu-15 and Glu-25 of the same subunit or to Glu-15 and Glu-4 of the adjacent subunits. Divalent agonists, such as succinylcholine and decamethonium, may bind to the α -subunit Glu-15 of the open conformation and to Glu-47 of the γ or δ subunit or to Glu-15 of the β subunit.

Other Conformations. Because of the many degrees of freedom in folding polypeptide chains and in positioning subunits next to each other, it is unlikely that

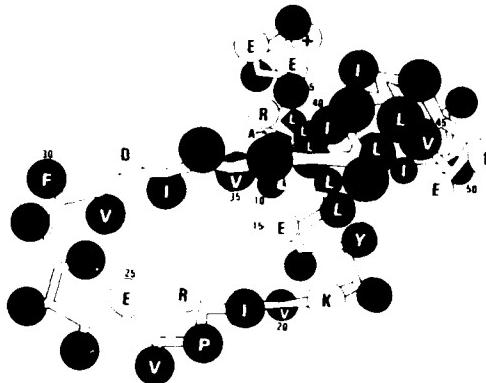


Fig. 12. Model II of α -subunit tertiary structure. See text for details.

every aspect of the proposed models is correct. The least ambiguous part of the models is the tertiary structure of the portion (residues 15 to 49) of the α subunit that bind to the snake neurotoxins. Even portions of this segment are not certain; e.g., there are other ways of folding the sections 23–36 and 39–56 that will bind the toxin as well as the model presented here.

An alternative structure of the α subunit is shown in Fig. 12. The bonds that this structure would make with the neurotoxins are listed in Table II. This model (Model II) is similar to the original model (Model I) in that (1) the initial segment is an α helix and other parts of the secondary structures are the same, (2) Glu-15 and Glu-25 bind to the same positively charged groups on the toxins, and (3) the backbone of a β segment binds to the backbone of one of the central β strands of the snake neurotoxins. The tertiary structures, however, are quite different. Model II agrees well with the predictions of the secondary structure, but it forms fewer bonds with the neurotoxins.

Model II α subunits can form a dimeric complex that, like Model I, will bind two snake toxins so that their "tails" meet in a way that mimics alloferin (see Fig. 13). Alloferin should bind well to the center of this complex since its aromatic rings fit over the aromatic Tyr-17 side chains, its hydroxyl groups bind to the Gln-38 amide groups, and its positively charged groups fit near the negatively polar C-terminal end of the α -helix and Glu-15 carboxyl groups. Formation of a channel between two Model II subunits is more difficult to envision; however, this is not a stringent requirement since it is not supported by any experimental data. Like Model I, conformational changes can be envisioned that involve movements of the α helices; e.g., the two helices could swing down so that the hydrophobic groups of the two helices bind to each other and the carboxyl group of one Glu-4 side chain binds to the N-terminal amine of the adjacent helix. As with Model I, portions of Model II can be modified in a manner that should still allow it to bind the neurotoxins; e.g., sections 23–29 can be given an α -helix conformation or sections 30–56 can be altered so that the backbone of sections 32–40 binds to the backbone of sections 34–42 of α cobra toxin and erabutoxin b and

Table II. Bonds Between Snake Neurotoxins and Model II of the α -Subunit*

	α Cobra toxin	α Subunit	Erabutoxin b	α Subunit
Salt bridges	K-12 K-23 D-27 R-33 R-36 D-38	D-32 E-25 K-19 E-15 E-15 R-22	K-15 K-27 D-31 R-33 E-38	D-32 E-25 K-19 E-15 R-22
Hydrogen bonds	Y-21 K-23 bb 30-33 S-31 bb 34-42	T-28 T-28 bb 37-40 Q-38 bb 37-29	K-27 bb 32-33 bb 34-43 Q-8 bb 44-47 T-47	T-28 bb 37-28 bb 37-28 T-34 bb 27-24 H-26
Hydrophobic interactions	W-25 F-28 I-32 C-41 & C-14	I-21 Y-17 L-12 & L-39 F-30	W-25 F-32 I-36 C-41 & C-17	I-21 Y-17 V-35 F-30 P-50

*Shown in Fig. 12. Notation is the same as in Table I.

the backbone of sections 49-55 binds to sections 8-14 of α cobra toxin and sections 11-17 of erabutoxin b.

Finding that the same sequence can be placed in quite different tertiary structures that are consistent with the secondary structures and that should strongly bind the neurotoxins indicates the limitations of the approach. It also stresses the importance of obtaining better structural data and developing better methods of predicting the structure.

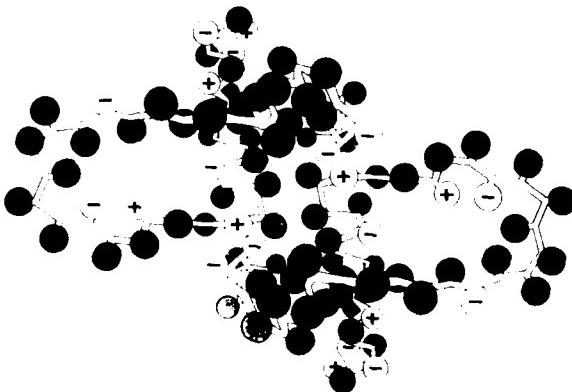


Fig. 13. A dimeric complex of two Model II α subunits. Alloferin would bind in the center between the two subunits. See text for details.

the free sulfhydryls without altering the binding of the toxin. It may be possible to use this method to bind together two neurotoxin molecules. From the model, one can predict the connecting chain lengths that should be required to allow both toxin molecules to bind to the various conformations. When both toxin molecules bind, the total number of bound toxins should be only half the value obtained when only one molecule binds, and the binding affinity should be higher. Alternatively, the distance between the tails of bound neurotoxins may be determined by attaching to the sulfhydryl group fluorescent or spin probes that interact with each other.

Many features of the models described here were developed by constructing Nicholson or CPK molecular models of the proposed structure and examining possible ways that the toxins or drugs could interact with various sites. This method appears satisfactory for large molecules, such as the snake neurotoxins, that bind very strongly and have many points of interaction. It is less satisfactory for small molecules like the agonists because of the small number of interactions, the degrees of freedom that one has in positioning the protein side chains and altering the conformations of the agents, and the nonquantitative nature of the approach. Assuming that the positively charged group of agonists and antagonists binds to Glu-15 of the α subunit, it is still difficult to use this approach to determine which of the possible conformations is most likely to be correct. Showing that agonists could bind to a given conformation is a necessary condition, but it is certainly not sufficient. For this type of analysis to be convincing, a more quantitative approach is needed that can evaluate the binding constants of a series of drugs to each conformation. Several groups are attempting to develop systems to evaluate interactions between drugs and their receptors (for review, see Gund *et al.*, 1980); however, it is not apparent that these approaches are sufficiently quantitative to correctly predict which of the possible models is most likely to be correct. In spite of the uncertainties, the models predict the positions of various groups on the receptor fairly well. It thus may be feasible either to synthesize new compounds or to modify existing compounds that should bind to specific sites on the AChR with a high affinity. If the agents bind covalently, it may be possible to identify the subunit and residue to which they bind.

Covalently bound affinity-labeling agents and cross-linking agents could help determine which subunits are next to each other. Raftery *et al.* (1979) have shown that a photolabeling agent that is covalently bound to a sulfhydryl on the tail portion of α -bungarotoxin will bind covalently to the δ -subunit. This result is consistent with the model presented here, although it is not apparent why the γ subunit is not labeled also.

One of the main purposes of this discussion is to emphasize the importance of the determination of the entire sequence of all of the subunits. Although Raftery *et al.* (1980) have made a good start, there is difficulty in isolating large quantities of the protein and in analyzing the sequence of large, insoluble proteins. The recent identification of the AChR messenger RNA suggests an alternative approach in which the complementary corresponding DNA is synthesized and the DNA is then sequenced (Mendez *et al.*, 1980). Thus the prospects of eventually obtaining the entire sequence are fairly good.

Obviously, the determination of more of the sequence of the subunits would be helpful in analyzing more of the AChR structure and in eliminating some of the

DISCUSSION

The main finding of this paper is that the polypeptide chains of residues 15 to 49 of the α subunit can be folded so that it should bind the snake neurotoxins with a high affinity. The implications of these structures on aggregation of the AChR subunits, the agonist and antagonist binding, AChR gating, and overall structure of the subunits were examined. These implications are secondary and more speculative than the tertiary structure of the α subunits. It is important to recognize that the models presented here are working hypotheses and that it is unlikely that every aspect of the models is correct. The main purpose of the models is to aid in the design of experiments that will test aspects of the models.

Before a great deal of time and energy is spent on testing precise details of the models, it is prudent to test more general features. One of the most crucial tests of the model is to determine whether the N-terminal segments are part of the extracellular soluble domains and, if so, whether they form the postulated binding sites. In a number of experiments, side chains near the cholinergic or neurotoxin-binding sites have been covalently labeled. By analyzing the sequence of labeled AChRs, it may be possible to identify the portions that comprise the receptor-binding sites. These types of experiments could be facilitated by enzymatically cleaving the subunits into identifiable peptides and then determining which peptides contain the labels and/or whether any of the peptides will bind the drugs and toxin. An initial step in this direction has already been taken. Trypsin treatment of isolated AChRs can be used to separate the α subunit into two domains: a 27,000-dalton soluble domain that binds neurotoxins, agonists, and competitive inhibitors (Bartsfeld and Fuchs, 1979), and a membrane domain that can be selectively labeled with [5-¹²⁵I]iodonaphthyl-1-azide when the AChR complex is in the membrane (Tarrab-Hazdi *et al.*, 1980). If the model proposed here is correct, the soluble domain should have the same N-terminal sequence as the entire subunit.

An alternative approach of determining whether the N-terminal segments form the receptor-binding sites is to isolate or synthesize the N-terminal segments, make antibodies to these segments, and determine whether the antibodies bind to the extracellular AChR domains and, if so, whether they inhibit the binding of the neurotoxins, agonists, or antagonists to the AChR. Also, if antibodies are found that inhibit the binding of these agents to AChRs, their binding and that of agonists and antagonists to the N-terminal segments could be analyzed.

If it is shown that the N-terminal segments form the cholinergic binding sites, one must determine whether any of the conformations suggested here are correct. If it can be shown that the N-terminal segments or some other segment still has the structural integrity to bind neurotoxins and/or cholinergic agents, then structural analysis of these segments could be informative. It is probable that the soluble domains of AChRs can be crystallized more easily than the entire subunit. However, the crystalline structure of portions of the subunits must be interpreted with care because of possible differences in the conformations.

The proposed mechanism of binding of the snake neurotoxins may be testable. Raftery *et al.* (1979) have found that the disulfide bond of the tail portion of α -bungarotoxin can be reduced and that a molecule can be covalently bound to one of

ambiguities of the model proposed here. Affinity-labeling experiments indicate that reduction of a disulfide bond on the AChR leads to the exposure of an α -subunit sulfhydryl group that is near the cholinergic binding site (Karlin, 1969; Damle and Karlin, 1980). Thus, one hopes that determination of more of the sequence N terminal will reveal a sulfhydryl group that can be positioned near the proposed agonist-binding site without altering the model conformations. It is quite feasible that this would be possible for only one of the alternative conformations described here.

The model presented here has not dealt seriously with conformation of the transmembrane domain other than to suggest that the channel is formed between the subunits. However, transmembrane domains may be more appropriate for the model-building approach than are soluble domains. There are theoretical reasons to believe that the hydrophobic environment of the lipid phase will impose considerably more order (i.e., more α -helix and β -sheet structures and more regular packing of these structures) than is generally observed in soluble proteins (Kennedy, 1978). This hypothesis is supported by the very regular structure of bacteriorhodopsin (Unwin and Henderson, 1975; Engelman *et al.*, 1980). Because the protein is in a membrane, labeling agents can be used to identify residues and segments that are in contact with the lipid phase, the extracellular aqueous phase, and the intracellular aqueous phase, and residues that become exposed when the channel opens. Labeling agents have already been used to show that portions of the α -subunit are in contact with the membrane lipid (Tarrab-Hazdai *et al.*, 1980) and that the δ subunit of *Torpedo marmorata* is near and/or comprises the local anesthetic-binding site (Saitoh *et al.*, 1980). In designing a model of the transmembrane protein structure, one can also use the structure of the putative channel-blocking drugs, the size and nature of the various cations that will and will not pass through the channel, and the voltage dependence of activation and desensitization kinetics. If membrane fragments can be isolated that have present only the transmembrane portions, then X-ray diffraction, electron microscopy, or other techniques that give structural information could be informative. Additional constraints on the model would be suggested by similarity of the amino acid compositions and sequences of the transmembrane portions to those of gap junction channels or other channels for which the protein structure is better defined.

This paper is intended to indicate the role that model building may play in determining the structure of the AChR complex. With current progress in analyzing AChR complexes, data should be available soon to allow the design of models less speculative than those presented here. Progress is also being made in the development of methods to predict the secondary and tertiary structure of proteins from their sequences (Cohen *et al.*, 1980), in modeling structures of other membrane proteins (Engelman *et al.*, 1980; Guy, 1980), and in predicting more precisely how drugs bind to proteins (Gund *et al.*, 1980). Thus it may be possible to predict the structure of the AChR complex without having precise X-ray diffraction data.

Biochemical and structural analysis of the nicotine AChR complex is made possible primarily because of its abundance in the electric organs of rays and eels. Most postsynaptic receptors and channels are not as easily analyzed. It is quite likely, however, that most postsynaptic receptor complexes evolved from the same protein and that they have similar structural features. This concept is supported by the finding that several postsynaptic channels that are activated by different transmitters are

blocked by the same drugs (Carpenter *et al.*, 1977). Determination of the structure of the nicotinic AChR complex may thus be important in the design of molecular models of other postsynaptic channels. For example, by changing side chains on the general backbone structures of portions of the AChR, one may be able to design molecular models of other types of receptors and channels that account for differences among the pharmacology, gating kinetics, and ion selectivities of the channels.

SUMMARY

(1) The N-terminal sequence of the first 54 residues of the four subunits that comprise the acetylcholine receptor (AChR) complex of *Torpedo californica* were determined recently (Raftery *et al.*, 1980). The aim of this paper is to examine the hypothesis that these segments form the binding sites for the cholinergic snake neurotoxins, agonists, and competitive inhibitors.

(2) Nicholson molecular models were constructed of the structures of erabutoxin b, α -cobra toxin, various agonists and antagonists, and the N-terminal segments of the AChR subunits. The conformations of the AChR subunits were influenced by theories that predict the secondary structure from the sequence, the requirement that the structures bind the agonists and antagonists with the appropriate stoichiometries, the requirement that most of the apolar side chains be buried in the interior of the structure, and the dimensions and spatial arrangements of the AChR indicated by electron microscopy studies.

(3) Subunit conformations were found that were consistent with the hypothesis that the cholinergic binding sites are formed by the N-terminal segments of the α subunit. The models suggest mechanisms by which opening of the channels is triggered and the AChR complex desensitizes. For some portions where the structure is less certain, a number of alternative conformations are suggested.

(4) The proposed models serve as excellent working hypotheses for the design of experiments to examine the AChR structure. By testing the hypotheses, by obtaining more structural data on the AChR, and by improving the methods of analyzing the AChR structure, it may be possible to determine the structure and functional mechanisms of the AChR without having precise X-ray crystallographic data.

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